Development of pre-columnar derivatization method of glutathione reduced by dansyl chloride for its determination by reversed-phase high-performance liquid chromatography method

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ABSTRACT

Aim: The present study is devoted to the development of the methodology for the analysis of restored glutathione by pre-columnar derivatization with dansyl chloride. Materials and Method: Glutathione is a tripeptide (L-glutamyl-L-cysteinyl glycine), which performs the most important functions in living organisms. As a source of active sulfhydryl group, it provides antioxidant protection of cells and is the factor of xenobiotics conjugation. Currently, this molecule is considered as a promising therapeutic agent. Result and Discussion: Therefore, it is necessary to create an optimal analytical base for the development of pharmaceutical facilities on its basis. Since glutathione does not have the necessary spectral characteristics for direct analysis, the methodology for the determination of glutathione was developed on the basis of this by pre-columnar derivatization with dansyl chloride using reversed-phase high-performance liquid chromatography method. Dansyl chloride acts as a chromo-shaped marker, according to which a modified molecule of glutathione can be analyzed. Dansyl chloride as a derivatizing agent is chosen because of its safety and availability. The resulting dansylates are extremely stable. The derivatization is a direct interaction of glutathione with dansyl chloride in alkaline medium. The detection of the resulting derivatives was carried out by the absorption in ultraviolet light using a diode array detector. It has been established that two derivates with different spectral data are developed during the interaction of glutathione with dansyl chloride. The division of derivatives was carried out in the mode of gradient elution. Conclusion: This technique was also evaluated for the possibility of glutathione quantitative determination. The sensitivity of this procedure was 0.001% or 3.2 × 10⁻⁵ moles. The linear relationship between the analytical signal (peak area) and concentration was observed in the range of 0.001–0.04%, the correlation coefficient made 0.9978.

KEY WORDS: Dansyl chloride, Derivatization, Restored glutathione, Reversed-phase high-performance liquid chromatography

INTRODUCTION

Glutathione (γ-L-glutamyl-L-cysteinyl glycine) is the most important low molecular weight intracellular thiol tripeptide synthesized by almost all animal cells. Its role is to provide a number of important biological functions. Due to the sulfhydryl group of cysteine, it participates in the reactions of restoration and conjugation. Using these reactions, peroxides and many xenobiotic compounds are removed.[1] The reactions of peroxide elimination take place due to the fact that glutathione serves as the co-factor of glutathione peroxidase enzyme, while preventing the oxidation of free thiol groups of important proteins, including enzymes, the peroxidation of phospholipids in cell membranes is reduced.[2] The removal of xenobiotic compounds is carried out by direct conjugation with glutathione and the subsequent secretion of the adduct from the cell.[3] Thus, this molecule forms the basis of intracellular redox status, thereby protecting cells from active forms of oxygen.[4]

The described biological effects of glutathione single out its potential therapeutic activity in the correction of numerous nosologies and indicate the perspective of drugs creation based on it. To develop the optimal...
pharmaceutical forms of glutathione, it is necessary to develop an effective method for its analysis.

A number of analytical methods have been described in the scientific literature for the analysis of glutathione. It should be noted that all modern analytical approaches to the determination of glutathione are based on the use of liquid chromatography (LC) with various detectors.

To identify glutathione conjugated with proteins, LC is used in combination with mass spectrometry (MS) and tandem MS to detect it as bound or free forms in organs and cells.[5]

Ethacrynic acid and its methyl ester have been proposed in biological samples as pre-chromatographic derivatization reagents for high-performance LC (HPLC) analysis of glutathione.[6]

Due to the presence of the sulfhydryl group, glutathione readily reacts with o-phthalic aldehyde in the presence of ethyl ester of N-acetylcysteine to develop a highly stable and fluorescent isodole derivative. This allows to perform its quantitative determination in biological systems, in particular in erythrocytes, by HPLC method with fluorescence or photometric detection.[7] A similar method is used to determine nitrosoglutathione.[8]

Since glutathione is the marker of oxidative stress, then it is determined by derivatization with the alkylation agent N-ethylmaleimide in blood and other tissues. It prevents the auto-oxidation of glutathione. Then, the conjugate is determined by HPLC[9] or by LC-tandem MS.[10] This approach is also used to determine glutathione in the striatum of Wistar rat brain.[11]

The comparative analysis of glutathione determination in human blood was performed using LC in combination with tandem MS (LC-MS/MS) and HPLC with electrochemical detection (HPLC/ECD). The LC-MS/MS method showed the advantages in selectivity, accuracy, and sensitivity as compared to HPLC/ECD.[12]

2,3-naphthalene dicarboxaldehyde was also used to derivatize glutathione, which reacts rapidly with glutathione and its precursor, γ-glutamylcysteine, producing high-fluorescent derivatives. Unlike other aldehydes, the addition of an additional nucleophile is not required in the reaction with 2,3-naphthalene dicarboxaldehyde. The spectral properties of the product are suitable for its determination by the means of a fluorimetric detector. This fluorescence mark was used to determine glutathione in neurobiologic samples by capillary electrophoresis.[13]

The methodology for the quantitative determination of glutathione in pharmaceutical preparations was developed by the method of highly efficient LC on the reverse phase using ultraviolet (UV) detection. The validation of the method was evaluated in six categories: Linearity, range, detection limit, quantification limit, accuracy, and selectivity. The detection limit and the quantitative limit were 0.6 and 1.8 μg/ml, respectively.[14]

Since the decrease of glutathione level is associated with renal insufficiency, the method has been developed for the determination of this substance in hemodialysis patient erythrocytes. The erythrocytes were hemolyzed, deproteinized, derivatized with 5,5-dithio-bis (2-nitrobenzoic) acid, and analyzed using HPLC at gradient elution.[15]

To determine glutathione in human plasma, HPLC method was used with pre-columnar derivatization of 7-flouro-4-nitrobenzo-2-oxa-1,3-diazole. The separation of derivatized glutathione was carried out using a mobile phase consisting of phosphate buffer (0.02 mol/l, pH 6.0) - acetonitrile (77:23). Eluted derivatives were detected fluorometrically at an excitation wavelength of 470 nm and the wavelength of 530 nm.[16]

Glutathione and glutathione disulfide determination method were developed in yeast using the HPLC method after preliminary derivatization with dansyl chloride. Derivatization was carried out in the tetraborate buffer solution (pH 9.5) at the temperature of 60°C for 60 min. The reaction was stopped by the addition of glacial acetic acid. The resulting derivative was chromatographed in an isocratic mode of elution, and the detection wavelength was 254 nm.[17]

The described series of glutathione analysis methods mainly concerns its direct determination in biological objects; however, they are of little use for purposes of pharmaceutical analysis.

Based on the foregoing, the purpose of this study was to develop an optimal methodology for glutathione analysis, which allows to evaluate its quality in pharmaceutical facilities.

**Study Objects and Methods**

Restored glutathione was used as the object of the study (CAS No. 70-18-8, EC No. 2007254, Applichem, Germany).

Chromatographic separation was carried out using “Agilent Technologies 1200 Infinity” (USA) with an automatic sampler Agilent 1200, vacuum microdegasifier, gradient pump, and thermostat. Electronic spectra were recorded using Agilent 1200 diode matrix detector (the wavelength range made 190–950 nm, the cuvette with an optical path length of 10 mm, and the volume made 13 μl), the scanning step made 2 nm.
A steel chromatographic column Ascentis express C_{18}, 2.7 μm × 100 mm × 4.6 mm was used for tests.

1% aqueous solution of formic acid was used as the mobile phase (A), and ethyl alcohol was used as the phase (B). The elution was carried out under the following conditions: Flow rate: 0.5 ml/min, column temperature: 35°C, detection: 284, 296 nm, and sample volume: 1 μL.

The elution was carried out in the gradient mode as shown in Table 1.

Glutathione is the molecule that does not have significant chromophore fragments, suitable for the study by UV spectroscopy and HPLC with diode-matrix detection. Due to the lack of intrinsic absorption in the UV region of the spectrum, we developed the analysis of glutathione by reversed-phase (RP)-HPLC method using the means of its chemical modification. The analysis consists in the use of pre-columnar derivatization of the molecule studied with dansyl chloride. At that, a direct chemical reaction of glutathione with dansyl chloride in an alkaline medium takes place. Thus, the structure of glutathione, undergoing a chemical transformation, acquires a chromophore mark, the signal of which can be detected by a diode array detector during HPLC analysis.

The reasons of dansyl chloride derivatizing agent selection are that this reagent is not toxic, it is available, and the reaction of amino acids with it is rapid one. The resulting amino acid dansylates are extremely stable, have high light absorption and fluorescence, and therefore, can be analyzed by RP-HPLC using diode-matrix or fluorimetric detectors.[18,19] In addition, the degree of resulting derivative hydrophobicity has a wide range so that they can be separated during gradient elution in one chromatographic distillation.

**Glutathione Derivatization Method**

The solutions of dansyl chloride and glutathione were prepared to obtain the dansyl derivative of glutathione.

Such solvents as acetone, acetonitrile, and methanol were used to prepare the solution of dansyl chloride. In acetone, dansyl chloride showed limited solubility, so further they used acetonitrile or methanol.

Thus, to prepare dansyl chloride, about 0.025 g of dansyl chloride (an accurate weighed portion) was placed in a 25 ml volumetric flask, 10 ml of acetonitrile or methanol was added, mixed until the solution was completely dissolved, and the volume of the solution was brought to the mark with the same solvent and mixed.

Since the reaction of amino acid interaction with dansyl chloride occurs according to the type of electrophilic substitution, an alkaline reaction of the medium is required for this reaction. Therefore, 0.05 m of sodium tetraborate solution was used to prepare the glutathione solution. 0.1 g of the test glutathione sample was placed in a volumetric flask, 20 ml of 0.05 m sodium tetraborate solution was poured into volumetric flask of 100 ml, stirred until complete dissolution, the solution was brought to the mark with the same solvent, and the mixture was stirred.

2 ml of the prepared glutathione solution was placed in a 25 ml volumetric flask, and 2 ml of 0.1% dansyl chloride solution was added and shaken. The resulting solution was brought to the mark of 0.05 m by sodium tetraborate solution, stirred and heated in a drying oven at 80°C for 30 min. At the end of this period, the solution was cooled to room temperature and chromatographed under the above conditions.

**RESULTS AND DISCUSSION**

In the course of the described experiment, the chromatograms of dansyl chloride and dansyl derivatives of glutathione were obtained.

The reagent dansyl chloride in this system is characterized by the presence of one peak with the retention time of 6.3 min. UV spectrum of dansyl chloride is observed at the wavelength $\lambda_{\text{max}} = 284$ nm [Figure 1].

The chromatogram of glutathione derivatives [Figure 2] also shows the peak of a free reagent with a similar peak.

During the derivatization of glutathione, two derivatives are observed with retention periods of 8.4 min and 18.2 min.

UV spectra of the resulting derivatives differ somewhat from each other. As can be seen on Figure 3, the derivative I had a maximum absorption at $\lambda_{\text{max}} = 284$ nm, and the derivative II, $\lambda_{\text{max}} = 296$ nm.

Apparently, the development of two derivatives is conditioned by the fact that glutathione exists in two isomeric forms, which forms these derivatives.

The calculations of fitness parameters of the used chromatographic system are given in Table 2.

**Table 1: Conditions for the gradient elution of glutathione derivatives**

<table>
<thead>
<tr>
<th>Time, min</th>
<th>A, %</th>
<th>B, %</th>
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<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>10</td>
</tr>
<tr>
<td>60</td>
<td>0</td>
<td>100</td>
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The results of fitness criteria calculation (N > 5000, Rs > 1.5, Tf < 2), listed in Table 2, fit into EF recommended fitness parameters. Thus, the presented chromatographic system can be recognized as an acceptable one for the determination of glutathione derivatives. Furthermore, from the table presented, it can be concluded that the derivatives of glutathione are formed in the ratio of 3:1.

To assess the feasibility of this technique use for the quantitative determination of glutathione, a direct relationship was established between the concentration of the dansylated glutathione derivative and the analytical signal (peak area). For this purpose, a number of six calibration solutions of the dansylated glutathione were prepared in the following range of concentrations: 0.001–0.04%. The resulting calibration solutions were chromatographed in the abovementioned conditions. Based on the results of chromatography, a calibration graph was developed concerning the dependence of glutathione derivative concentration on the peak area [Figure 4].

The detectable minimum for the determination of glutathione derivative makes 0.001% (0.01 mg/ml or 3.2 × 10^{-5} mol).

In the indicated range of concentrations, the calibration dependence was rectilinear one. The regression equation was the following one:

\[ y = 12597x + 16.545 \]

The correlation coefficient was 0.9978, which indicates the presence of a linear relationship between the peak area and the glutathione derivative content.

### CONCLUSION

In the course of the performed studies, the method of glutathione determination was developed using pre-columnar derivatization with dansyl chloride by RP-HPLC. In this case, two derivatives with different spectral data are formed. The separation of derivatives is most expediently carried out in a gradient elution
The sensitivity of the determination was 0.001% or $3.2 \times 10^{-5}$ moles. The rectilinear relationship between the analytical signal (peak area) and the concentration was observed in the range of 0.001–0.04%.

Thus, the method of analysis was obtained which allows to assess the quality standards of glutathione in pharmaceutical facilities, including the developed pharmaceutical forms on its basis.

REFERENCES